Dominant-negative Gα subunits are a mechanism of dysregulated heterotrimeric G protein signaling in human disease

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Auriculo-condylar syndrome (ACS), a rare condition that impairs craniofacial development, is caused by mutations in a G protein–coupled receptor (GPCR) signaling pathway. In mice, disruption of signaling by the endothelin type A receptor (ETAR), which is mediated by the G protein (heterotrimeric guanine nucleotide–binding protein) subunit Gα11, and subsequently phospholipase C (PLC), impairs neural crest cell differentiation that is required for normal craniofacial development. Some ACS patients have mutations in GNAI3, which encodes Gα3, but it is unknown whether this G protein has a role within the ETAR pathway. We used a Xenopus model of vertebrate development, in vitro biochemistry, and biosensors of G protein activity in mammalian cells to systematically characterize the phenotype and function of all known ACS-associated Gαq mutants. We found that ACS-associated mutations in GNAI3 produce dominant-negative Gαq mutant proteins that couple to ETAR but cannot bind and hydrolyze guanosine triphosphate, resulting in the prevention of endothelin-mediated activation of Gαq/11 and PLC. Thus, ACS is caused by functionally dominant-negative mutations in a heterotrimeric G protein subunit.

INTRODUCTION

Heterotrimeric guanine nucleotide–binding proteins (G proteins) are gatekeepers of signal transduction that play critical roles in physiology. They cycle between inactive [guanosine diphosphate (GDP)–bound] and active [guanosine triphosphate (GTP)–bound] states to control the flow of information from extracellular cues to intracellular effectors (1–4). Resting heterotrimeric G proteins are composed of a GDP-bound Gα subunit in complex with Gβγ. G protein activation is predominantly carried out by G protein–coupled receptors (GPCRs), which promote the exchange of GDP for GTP on Gα (1, 3). This leads to the dissociation of the heterotrimer into Gα-GTP and free Gβγ, both of which act on downstream effector molecules. Gα subunits are classified into four major families, Gαs, Gαq, Gα11, and Gα12/13, which are characterized by their ability to modulate different effector molecules and second messengers. For example, Gα11 subunits dampen the production of cyclic adenosine monophosphate by inhibiting adenylyl cyclase, whereas Gαq subunits increase intracellular Ca2+ by activating phospholipase C (PLC). The evidence that the dysregulation of heterotrimeric G protein signaling causes various diseases is mounting rapidly, but the underlying molecular mechanisms in many of these diseases are elusive. Most G protein–associated mutations have been identified in genes encoding Gα subunits (5, 6) and are broadly classified as loss-of-function or gain-of-function mutations. Loss-of-function mutations in various Gα proteins are associated with the congenital diseases Albright’s hereditary osteodystrophy (Gαs), Nougaret night blindness (Gα11), and two types of dystonia (Gα14) (5, 7–10). On the other hand, gain-of-function mutations are somatic and exert a dominant effect by rendering the Gα subunit constitutively active. The most frequent alteration associated with gain-of-function mutations is guanosine triphosphatase (GTPase) deficiency, as seen in aberrant Gαs, Gαq, and Gα11 in up to 80% of patients with some types of cancer (6).

Auriculo-condylar syndrome (ACS) (11–13) is a rare condition that impairs craniofacial development. Evidence from genetic studies in humans and animal models indicates that ACS is caused by disruption of an endothelin type A receptor (ETAR)/Gα/PLC pathway that induces the expression of genes encoding the distal-less homeobox (DLX) transcription factors DLX5 and DLX6 required for specification and patterning of neural crest cells during craniofacial development (14). In humans, this hypothesis is supported by the identification of mutations in the genes encoding for the natural ETAR ligand endothelin–1 (ET-1) (15) and the Gαq/11 effector PLCβ4 (11, 12). This is in agreement with evidence in mice showing that knockout animals lacking Gαq and its close homolog Gα11 display craniofacial defects that resemble ACS (16, 17). However, this model contrasts with the genetic evidence in humans; no Gαq or Gα11 mutations have been found in ACS patients to date. Instead, some ACS patients have mutations in another Gα subunit, Gαq3. Whether Gαq3 functions between ETAR and PLCβ4 and the mechanism of action by which Gαq3 mutations affect this pathway are not yet known.

ACS is classified as type I, II, or III based on the presence of mutations in GNAI3 (encoding Gα13), PLCB4 (encoding PLCβ4), or EDN1 (encoding ET-1), respectively. Five autosomal dominant mutations in Gα13 have been found in type I ACS (11–13). All five mutations affect conserved amino acid positions that cluster within the nucleotide binding pocket (Fig. 1 and fig. S1). It has been speculated that ACS-associated Gα13 mutants may behave as dominant-negatives (meaning it interferes with the function of the normal gene product) or as constitutively active G proteins (11, 13, 14). However, none of these hypotheses or the possibility of acting as loss-of-function mutants leading to haploinsufficiency has been formally tested. Here, we investigated the molecular basis of type I ACS by systematically characterizing the functional consequences of the five Gα13 mutations found in ACS patients using in vivo and in vitro approaches.
ACS mutations increase the frequency of developmental defects induced by the ectopic expression of Ga\textsubscript{13} in *Xenopus laevis* embryos

As a first approach to investigate the functional consequences of ACS mutations in Ga\textsubscript{13}, we performed experiments with *Xenopus laevis* embryos as a development-relevant model. For this, we took advantage of previous observations showing that ectopic expression of Ga\textsubscript{13} induces developmental defects in *X. laevis*. During normal gastrulation, embryo cell movements cause the appearance of a new hollow cavity, the archenteron, while simultaneously inducing the progressive removal of another, the blastocoel. In Ga\textsubscript{13}-injected embryos, this process is disrupted: the archenteron does not inflate and the blastocoel is not removed. The abnormal distribution of the internal cavities alters the rotation of the embryo and causes an inversion of its normal gravitational orientation (Fig. 2A). This phenotype can be easily scored at the neurula stage because the neural tube faces downward instead of upward. Although it is not known whether specific signaling pathways are disrupted by the ectopic expression of Ga\textsubscript{13}, this system provides a suitable platform to test whether Ga protein mutants induce a gain or loss of function in the context of embryonic development. First, we validated that ectopic expression of Ga\textsubscript{13} caused the same defects as those previously observed for Ga\textsubscript{13} (Fig. 2A). We reasoned that if the ACS-associated mutations induce loss of function in Ga\textsubscript{13}, their expression will not lead to a Ga\textsubscript{13} overexpression phenotype. We found that this is not the case because the ACS-associated Ga\textsubscript{13} mutants exerted the opposite effect; expression of any of the five mutants (G40R, G45V, S47R, T48N, and N269Y) increased the frequency of gravitational inversion in embryos compared to those expressing wild-type Ga\textsubscript{13}; this was observed at two different injected doses of mRNA (Fig. 2, B and C). Embryo bisections revealed that the gravitational inversion in ACS-associated Ga\textsubscript{13} mutants (of which S47R is shown as a representative in Fig. 2B) was accompanied by defective archenteron inflation and incomplete blastocoel removal, indicating that the underlying cause of the phenotype is the same as in wild-type Ga\textsubscript{13}-injected embryos. We ruled out that the different abundance of the Ga\textsubscript{13} mutant proteins compared to that of wild-type Ga\textsubscript{13} caused the difference in phenotype frequency by confirming their equal quantities in immunoblots of embryo lysates (Fig. 2C). Together, these results demonstrate that ACS mutations increase the frequency of developmental defects caused by ectopic expression of Ga\textsubscript{13}, indicating that the mutations provide Ga\textsubscript{13} with dominant properties rather than induce a loss of function.

ACS-associated Ga\textsubscript{13} mutants display defective GTP binding

To further characterize the functional consequences of the ACS mutations in Ga\textsubscript{13}, we investigated their biochemical properties in vitro. We obtained very poor or no yields of soluble proteins when we attempted to purify Ga\textsubscript{13} G40R, G45V, T48N, or N269Y from *Escherichia coli*, suggesting that the mutations compromise G protein stability. Only Ga\textsubscript{13} S47R was purified in sufficient quantities for biochemical studies. The ability of Ga\textsubscript{13} S47R to bind nucleotides was investigated by using a well-established assay that monitors G protein activation upon nucleotide binding by measuring its susceptibility to limited trypsinolysis. Briefly, inactive, GDP-bound Ga is readily digested by trypsin, whereas active Ga, generated by the binding of GTP mimetics guanosine 5′-O-(3′-thiotriphosphate) (GTP\textsubscript{S}) or GDP-AlCl\textsubscript{3}/NaF (GDP-AlF\textsubscript{4} ), adopts a conformation that is resistant to trypsin digestion outside of a short N-terminal sequence, which is cleaved off by trypsin (19). We found that Ga\textsubscript{13} S47R protection from trypsinolysis after incubation with GTP\textsubscript{S} or GDP-AlF\textsubscript{4} was markedly reduced compared to that of wild-type Ga\textsubscript{13} (Fig. 3A), indicating that the mutant cannot bind nucleotides and/or it cannot change conformation efficiently upon GTP binding. Next, we investigated whether the S47R mutation affects the steady-state GTPase activity of Ga\textsubscript{13} and found that it was virtually abolished (Fig. 3B). The cause of this defect was failure to bind GTP, as determined by two independent approaches that measure binding of the nonhydrolyzable analog GTP\textsubscript{S} (Fig. 3, C and D). To elucidate whether the impaired activation of Ga\textsubscript{13} S47R was due to a defect specific to GTP binding or to nucleotide binding in general, we analyzed its nucleotide content by high-performance liquid chromatography (HPLC). Ga\textsubscript{13} proteins were exchanged into nucleotide-free buffer and rapidly denatured to release any bound nucleotide. The HPLC analysis revealed identical chromatograms for wild-type Ga\textsubscript{13} and Ga\textsubscript{13} S47R corresponding to GDP peaks of identical intensity (Fig. 3E). Together, these results demonstrate that Ga\textsubscript{13} S47R retains the capability to bind GDP but fails to bind GTP, rendering the G protein unable to switch into an active conformation.

Next, we investigated whether the GTP-binding defect of Ga\textsubscript{13} S47R is a common feature among all the ACS mutants. Because Ga\textsubscript{13} G40R, G45V, T48N, and N269Y mutants could not be purified from bacteria, we measured susceptibility to limited proteolysis after expression in mammalian cells. In initial experiments, we found that all the ACS mutant plasmids expressed lower protein quantities than wild-type Ga\textsubscript{13} in human embryonic kidney 293T (HEK293T) cells. To overcome this limitation and be able to make comparisons, we equaled the amount of protein expression by transfecting larger quantities of the corresponding Ga\textsubscript{13} mutants. Because Ga\textsubscript{13} S47R incubation was reduced for all five ACS-associated Ga\textsubscript{13} mutants (G40R, G45V, S47R, T48N, and N269Y) compared to that for wild-type Ga\textsubscript{13} (Fig. 3F). These findings indicate that defective GTP binding and G protein activation is a common feature of ACS-associated Ga\textsubscript{13} mutants.

ACS-associated Ga\textsubscript{13} mutants cannot be activated by a GPCR

Our in vitro data indicate that ACS mutations abolish the spontaneous exchange of GDP for GTP on Ga\textsubscript{13}. Therefore, we investigated whether
Gα for GPCR-catalyzed activation, we first examined whether ACS-associated Gbg also affect GPCR-catalyzed Gα of G protein exchange factors (GEFs) that accelerate the exchange of Gαi3 into Gα-GTP and Gβγ. Because association of Gα with Gβγ is an obligatory requirement for GPCR-catalyzed activation, we first examined whether ACS-associated Gαi3 mutants bind to Gβγ in cells. For this, we used a cell-based bioluminescence resonance energy transfer (BRET) system that monitors the association of free Venus-Gβγ (BRET acceptor) with the C-terminal fragment of its effector, G protein–coupled receptor kinase 3 (GRK3), fused to a modified luciferase (masGRK3ct-Nluc, BRET donor) (20, 21). In the absence of Gα, free Gβγ robustly associates with the GRK3 probe, leading to high BRET signals, whereas expression of Gα subunits favors the formation of Gα:Gβγ complexes and diminishes the association of Gβγ with GRK3, thereby quenching the BRET signals (fig. S2A). As expected, we found that ectopic expression of wild-type Gαi3 in HEK293T cells decreased the Gβγ:GRK3 BRET signals compared to cells not transfected with Gαi3 (fig. S2B). When we transfected cells with plasmids expressing ACS-associated Gαi3 mutants G40R, G45V, S47R, T48N, or N269Y at the appropriate quantities to obtain protein amounts similar to wild-type Gαi3 (fig. S2B, lower panel), we found that the BRET signal was also significantly reduced compared to cells expressing Gβγ alone (fig. S2B, upper panel). However, we also observed that expression of some of the mutants (G40R, G45V, T48N, and N269Y) did not quench the BRET signal as efficiently as did expression of wild-type Gαi3 (fig. S2B, upper panel), which is indicative of reduced Gβγ binding. These results indicate that, although some of the ACS-associated Gαi3 mutants display mild to moderate Gβγ binding defects, all of them are capable of forming significant amounts of Gαγ complexes that serve as GPCR substrates.

We used the same BRET-based experimental system described above to monitor the kinetics of G protein activation upon stimulation of a prototypical Gβ-coupled GPCR, the adenosine 1 receptor (A1R) (Fig. 4A and fig. S2C). In cells expressing wild-type Gαi3, stimulation of A1R with adenosine resulted in a rapid increase of the BRET signal (Fig. 4B and fig. S2C), which is indicative of dissociation of Gβγ from Gαi3 upon G protein activation. This activation was reversed upon GPCR inhibition because addition of the A1R antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) returned BRET signals to basal values (fig. S2C). When analogous experiments were performed with cells expressing any of the five ACS-associated Gαi3 mutants (G40R, G45V, S47R, T48N, or N269Y) in protein amounts equivalent to wild-type Gαi3 (as in fig. S2B), the BRET increase upon adenosine stimulation was essentially absent (Fig. 4B). Because, under these conditions, some ACS-associated Gαi3 mutants displayed increased basal BRET values that are indicative of impaired association with Gβγ (fig. S2B), we performed additional experiments to rule out the possibility that the observed defect in GPCR-mediated activation was due to the impaired formation of Gαγ heterotrimers. For this, we adjusted the transfected amounts of wild-type Gαi3 and mutant plasmids to obtain similar basal BRET values for all of them, which reflect equivalent Gαγ heterotrimer formation. We found that, under these conditions, the BRET increase upon adenosine stimulation was absent for all the Gαi3 mutants. Because it is also possible that impaired activation of Gαi3 mutants by GPCRs is due to G protein mislocalization, we performed an additional control by investigating the subcellular localization of the Gαi3 mutants when expressed in the presence of Gβγ. We found that the subcellular distribution of each of the five ACS-associated Gαi3 mutants was similar to that of wild-type Gαi3 (fig. S3), including localization at the plasma membrane as well as endomembranes, as previously reported (22, 23), indicating that defective
activation by GPCRs is not due to G protein mislocalization. Together, these results demonstrate that trimeric G proteins bearing $\alpha_{13}$ subunits with ACS-associated mutations cannot be activated by GPCRs.

**ACS-associated $\alpha_{13}$ mutants have increased binding to GEFs**

The results presented so far indicate that ACS-associated $\alpha_{13}$ mutants exert a dominant effect in *Xenopus* bioassays, cannot bind GTP efficiently, and fail to be activated by the GEF activity of ligand-bound GPCRs. These results suggest that these ACS-associated $\alpha_{13}$ mutants may work as dominant-negative proteins by forming nonproductive complexes with GEFs because the dissociation that occurs upon GTP binding to the wild-type protein (24–27) would not occur for the mutants. If this is the case, we reasoned that ACS-associated $\alpha_{13}$ mutants would associate with GEFs better than the wild-type protein. We initially explored this idea with the nonreceptor GEF Ric-8A. Much like GPCR GEFs, Ric-8A binds with moderate affinity to GDP-bound G proteins, forms a high-affinity complex with the nucleotide-free G protein, and dissociates once the G protein binds GTP (Fig. 5A) (25–27). To validate that Ric-8A can be used as a tool to probe for different $\alpha_{13}$ conformations in yeast two-hybrid experiments, we used mutants that mimic different states of $\alpha_{13}$ along the activation pathway (Fig. 5A). As expected, binding of Ric-8A to $\alpha_{13}$ Q204L, a mutant constitutively bound to GTP (19), was diminished compared to wild type, which is predominantly bound to GDP. Moreover, binding was enhanced between Ric-8A and $\alpha_{13}$ N269D (Fig. 5, B and C), a synthetic “GEF-trapping” mutant that mimics the nucleotide-free intermediate state and forms a nondissociable complex with GPCRs (28). We found that, similar to the $\alpha_{13}$ N269D mutant, all five ACS-associated $\alpha_{13}$ mutants showed increased binding to Ric-8A compared to wild-type $\alpha_{13}$, suggesting that they assemble into more stable G protein–GEF complexes. Next, we performed communoprecipitation experiments to directly test whether ACS-associated $\alpha_{13}$ mutants bind with higher affinity to ET$\alpha$R, the GPCR that governs the signaling pathway dysregulated in ACS (14). We found that all five ACS-associated $\alpha_{13}$ mutants displayed increased binding (three- to fivefold) to myc-tagged ET$\alpha$R compared to wild-type $\alpha_{13}$ (Fig. 5D). Collectively, these results indicate that ACS-associated $\alpha_{13}$ mutants bind to GEFs with high affinity.

**ACS-associated $\alpha_{13}$ mutants exert a dominant-negative action on the ET-1/ET$\alpha$R/$\alpha_{13}$ pathway**

ACS is caused by disruption of the ET-1/ET$\alpha$R signaling axis (14). To further explore the idea that ACS-associated $\alpha_{13}$ mutants work as dominant-negative
Fig. 4. ACS-associated G\(_a_{3}\) mutants are not activated by the GPCR A\(_R\). (A) Schematic diagram depicting the BRET assay used to monitor the dissociation of G\(_a_{3}\)-G\(_b\) trimer upon GPCR stimulation. Under resting conditions, Venus-tagged G\(_b\) (V-G\(_b\)) associates with G\(_a_{3}\) and BRET signals are low. Upon stimulation of A\(_R\) with adenosine, G\(_i\) trimers dissociate, and free V-G\(_b\) binds to mas-GRK3ct-Nluc (GRK), leading to an increase of BRET signal. (B) Assessment of A\(_R\)-induced dissociation of G\(_i\) trimers containing G\(_a_{3}\) WT or ACS mutant subunits. HEK293T cells were transfected with plasmids encoding for Venus(155–239)-G\(_b\) (VC-G\(_b\)), Venus(1–155)-G\(_\gamma_2\) (VN-G\(_\gamma_2\)), mas-GRK3ct-Nluc, and A\(_R\) along with G\(_a_{3}\) WT or ACS mutants (G40R, G45V, S47R, T48N, or N269Y), and BRET was measured every second (as described in Materials and Methods). Equal amounts of V-G\(_b\) and the different G\(_a_{3}\) proteins were verified by immunoblotting (fig. S2). After 30 s under resting conditions, cells were stimulated with adenosine (1 \(\mu M\)). The average of BRET signal during the first 30 s (basal BRET) was subtracted from each data point to present the data as increase of BRET (\(\Delta\)BRET). One representative experiment of four is shown.

DISCUSSION

The main finding of this work is the identification of a new mechanism by which mutations in trimeric G proteins cause human disease. Our data indicate that ACS-associated G\(_a_{3}\) mutants are dominant-negative proteins that disrupt a G protein–dependent signaling pathway required for proper craniofacial development. This pathway consists of the activation of G\(_q\)-G\(_\gamma_2\)-ET\(_{\alpha}\), which in mice leads to activation of G\(_q_{11}\)-dependent signaling and subsequent transcriptional regulation of neural crest cell development (14). Although ET\(_{\alpha}\)R displays some promiscuity in terms of G protein selectivity, previous work (29) and our work here have shown that it preferentially activates G\(_q_2\) over G\(_q_{11}\). We propose that the dominant-negative action of ACS-associated G\(_a_{3}\) mutants is due to their inability to bind G\(_q_2\) (Fig. 7). The unproductive coupling of ACS-associated G\(_a_{3}\) mutants to ET\(_{\alpha}\)R is due to their inability to bind G\(_q_2\) and favor sustained association with ET\(_{\alpha}\)R even after ligand stimulation.

In this scenario, the availability of ET\(_{\alpha}\)R for G\(_q_2\) activation is diminished, and endothelin-dependent signaling important for craniofacial development is impaired.

Previous genetic studies in humans and animal models indicate that disruption of an ET\(_{1}\)/ET\(_{1}\)R/G\(_q_2\)/PLC\(_\beta_4\) pathway that controls craniofacial development leads to ACS (14). Two items support that G\(_a\) subunits of the G\(_q_{11}\) family fulfill the role of mediators in this pathway. One is that PLC\(_\beta_4\), contrary to the rest of PLC\(_\beta\) isoforms, is activated exclusively by G\(_q_1\)-related G\(_\alpha\) subunits and not by G\(_b\) subunits, which can originate from heterotrimers containing any type of G\(_a\) (30, 31, 32). The other one is that G\(_q_2\)/G\(_q_{11}\) double knockout mice display craniofacial developmental defects analogous to those found in ACS (16, 17). However, results from mouse models should be interpreted with caution because there is a precedent that the signaling mechanism that controls craniofacial development in mice might have differences with humans. For example, knockout mice lacking PLC\(_\beta_4\) do not display a craniofacial phenotype (32), whereas there is at least one reported case of autosomal recessive ACS caused by mutations in PLC\(_\beta_4\) (33). Moreover, no mutations in G\(_q_2\) and/or G\(_q_{11}\) have been found in ACS patients. Conversely, the presence of mutations in G\(_a_{3}\) indicates that this G protein plays a role in the signaling pathway disrupted in ACS, but the mechanism involved has
Fig. 5. G\textsubscript{ai3} ACS mutations increase binding to GEFs. (A) Schematic of Ric-8A binding to different conformations of G\textsubscript{ai3} during its activation cycle and description of constructs that mimic each one of these conformations. (B) Ric-8A binding by G\textsubscript{ai3} WT or ACS mutant using yeast two-hybrid assays (schematic, top; AD, Gal4 activation domain; BD, Gal4 DNA binding domain). Data are means ± SEM (n = 3 experiments: *P < 0.05 and **P < 0.01, Student’s t test). (C) Immunoblot of strains used in (B). (D) Relative affinity of ACS-associated G\textsubscript{ai3} mutants for the ET\textsubscript{AR} GPCR as determined by immunoprecipitation (IP) with myc or immunoglobulin G (IgG) antibody in lysates of HEK293T cells transfected with myc-ET\textsubscript{AR} and the indicated G\textsubscript{ai3} constructs. Blots are representative of three experiments.

Fig. 6. ACS-associated G\textsubscript{ai3} mutants prevent ET\textsubscript{AR}-mediated activation of G\textsubscript{aq}. (A and B) BRET assay in HEK293T cells transfected with G\textsubscript{aq} (left) or G\textsubscript{ai3} (right) along with V-G\textsubscript{bg}, mas-GRK3-Nuc, and ET\textsubscript{AR}, stimulated (arrow) with the indicated concentrations of ET-1, analyzed as in Fig. 4B. One representative experiment is shown in (A). Data in (B) are means ± SEM (n = 3 experiments). (C) Putative mechanism of dominant-negative action of ACS-associated G\textsubscript{ai3} mutants on G\textsubscript{aq} activation by ET\textsubscript{AR} as determined by BRET assays. In cells expressing G\textsubscript{ai3} WT (top), ET\textsubscript{AR} couples predominantly to G\textsubscript{ai3}. In cells expressing G\textsubscript{ai3} mutants (bottom), high-affinity binding of mutants to ET\textsubscript{AR} precludes G\textsubscript{ai3} activation. (D) HEK293T cells transfected with G\textsubscript{aq} (left) or G\textsubscript{ai3} WT (right) (1 μg) and substoichiometric amounts of G\textsubscript{ai3} WT or an ACS mutant (0.12 μg) along with V-G\textsubscript{bg}, mas-GRK3-Nuc, and ET\textsubscript{AR} were stimulated (arrow) with ET-1 (0.3 μM G\textsubscript{aq}/3 μM G\textsubscript{ai3}). One representative of three experiments, with respective immunoblots (below), is shown. HA, hemagglutinin.

remained unclear until now. Our results support that ACS-associated G\textsubscript{ai3} mutants disrupt signaling “horizontally” by blocking GPCR-mediated activation of another G protein, G\textsubscript{q}. Although it is possible that ET\textsubscript{AR}-G\textsubscript{ai3} signaling participates in craniofacial development, our results indicate that ACS-associated G\textsubscript{ai3} mutants do not exert their dominant-negative effect under conditions in which ET\textsubscript{AR}-G\textsubscript{q} signaling is efficiently inhibited (Fig. 6D).

The lack of dominant-negative inhibition on G\textsubscript{ai3} is somewhat puzzling because the unproductive coupling of ACS-associated G\textsubscript{ai3} mutants with ET\textsubscript{AR} would be expected to interfere with the binding of any G protein to this GPCR. However, the dominance of ACS-associated G\textsubscript{ai3} mutants could be different depending on the nature of the G\textsubscript{q}-ET\textsubscript{AR} and G\textsubscript{ai3}-ET\textsubscript{AR} interactions. It is possible that the dominant effect is dampened for G\textsubscript{ai3} if G\textsubscript{q} proteins have higher affinity for ET\textsubscript{AR} than G\textsubscript{ai3} and/or if G\textsubscript{aq} proteins, but not G\textsubscript{ai3}, “precouple” to ET\textsubscript{AR} before agonist stimulation. Although further investigation is required to clarify the specificity of the dominant-negative function of ACS-associated G\textsubscript{ai3} mutants, our results suggest that the primary mechanism by which they cause ACS is “horizontal” interference with G\textsubscript{q}-mediated signaling.

Another question that remains open is why ACS patients bearing mutations in G\textsubscript{ai3} do not display more pleiotropic phenotypes than the observed effect restricted to neural crest cell differentiation during development. It is possible that the dominant-negative function of ACS-associated G\textsubscript{ai3} mutants is dampened in other situations due to differences in overall G\textsubscript{ai3} expression. Because ACS-associated G\textsubscript{ai3} mutant proteins are intrinsically unstable, the dominant-negative function would become apparent only when sufficient amounts accumulate, for example, by increased gene transcription and/or posttranslational stabilization. Another possibility is that the dominant function is dampened in the presence of higher amounts of other G\textsubscript{q} subunits and/or when multiple GPCRs are present and activated simultaneously. Similarly, if the ACS phenotype is specifically associated with the disruption of G\textsubscript{q}-dependent signaling, the relative amounts of G\textsubscript{ai3} and G\textsubscript{aq} are bound to be critical for the development of ACS. In summary, obtaining additional information on the expression patterns of G\textsubscript{ai3}, G\textsubscript{aq}, and other signaling components in developing humans will be important to interpret the mechanistic results presented here.
In the past, the characterization of synthetic Go mutants with dominant-negative function has been instrumental in understanding the molecular basis of G protein regulation (28, 34–37). The present work is the first description of naturally occurring Go mutants with dominant-negative function, which demonstrates that this type of mutants is important not only as a research tool but also as an underlying cause of human disease. ACS-associated Go33 mutants have similarities and differences with some of the previously described synthetic dominant-negatives. For example, both an N→D mutant in Go G-4 box originally identified in yeast and Go, S43N (located in the G-1 box/P-loop) bind to Gβγ and have increased affinity for GEFs (28, 36, 38), much like Go33 in ACS. These mutants adopt a conformation that mimics an intermediate in the G protein activation cycle that traps GPCRs in an unproductive complex (28, 36). The positions mutated in Go N→D and Go, S43N correspond to N269D and S47R in Go33, two of the positions mutated in ACS. However, they are mutated to different residues, meaning N269Y and S47R, which may account for some of their different properties. The main difference is that Go N→D and Go, S43N are capable of binding GTP spontaneously (28, 36), whereas Go33 N269Y and S47R (as well as the rest of ACS mutants) do not. This unique feature of ACS-associated Go33 mutants is bound to contribute to their dominant-negative effect by precluding Go33 from adopting an active conformation and blocking the progression of the G protein cycle.

Another feature shared with other dominant-negative proteins like Go N→D is decreased stability (28, 38), which is most readily explained by their mimicry of an unstable nucleotide-free intermediary in the activation cycle (25, 39). All or four of five ACS-associated Go33 mutants expressed poorly in mammalian cells and bacteria, respectively. On the other hand, the amount of ACS-associated Go33 mutant proteins ectopically expressed in Xenopus or yeast was similar to wild-type Go33. This discrepancy is best explained by technical differences among experimental systems. G proteins were expressed at lower temperatures in Xenopus and yeast (16° and 30°C, respectively) compared to mammalian cells (37°C). In addition, the mutants were coexpressed with Ric-8A in yeast, which is a folding chaperone for Go (25). Regardless, these results suggest that ACS-associated Go33 mutants are potent dominant-negative proteins because they can efficiently disrupt signaling even when present at quantities lower than endogenous Go33 in mammalian cells.

As for other diseases caused by inherited G protein mutations like dystonias, Nougaret night blindness, or Albright’s hereditary osteodystrophy, the genetic pattern of type I ACS inheritance is dominant. However, our results indicate that the molecular basis for type I ACS dominant inheritance is different from other diseases caused by G protein mutants. In type I ACS, a single mutant allele is sufficient to cause the disease because of the dominant-negative action of Go33 variants, whereas in other diseases, a single G protein mutant allele causes haploinsufficiency. For example, primary torsion dystonia, craniocervical dystonia, or Albright’s hereditary osteodystrophy can be caused by nonsense or missense mutations that lead to absence of protein or severe protein structural defects (5, 9, 10). Nougaret night blindness is caused by a missense mutation in Go, but it has been unequivocally established that the disease arises from a loss of function not accompanied by any dominant-negative function (7, 8). Conversely, our results rule out haploinsufficiency as the cause of type I ACS because Go33 mutants provoke an increase rather than a decrease of the penetrance of Go33-dependent phenotypes in embryonic development assays (Fig. 2). On the basis of this finding, we conclude that type I ACS is the first human disease caused by G protein mutants that arises from a dominant-negative function.

MATERIALS AND METHODS

Reagents and antibodies

Unless otherwise indicated, all reagents were of analytical grade and obtained from Sigma or Fisher Scientific. The cell culture medium and the E. coli strain BL21(DE3) were purchased from Invitrogen. All restriction endonucleases were from Thermo Scientific, and E. coli strain DH5α was purchased from New England Biolabs. PfuUltra DNA Polymerase was purchased from Agilent. DPCPX, ET-1, and adenosine were from Sigma. [γ-32P]GTP and [35S]GTPγS were from PerkinElmer Life Sciences. Goat anti-rabbit and goat anti-mouse Alexa Fluor 680 or IRDye 800 F(ab’)2 were from LI-COR Biosciences. Rabbit antibodies raised against Go33 (C10) and Gβ (M-14) and mouse monoclonal antibodies against green fluorescent protein (GFP) (B-2) were from Santa Cruz Biotechnology. Rabbit polyclonal antibodies for myc tag were from Sigma (C9595). Mouse monoclonal antibodies raised against tubulin (12G10) and myc tag (9E10) were from the Developmental Studies Hybridoma Bank (University of Iowa), and the mouse monoclonal antibody for HA tag (12CA5) was from Roche.

Bioinformatics

The protein sequences of all 16 Go subunits in Homo sapiens were retrieved from UniProt, aligned using ClustalW, and shaded with BoxShade 3.21. Protein structure images were generated with PyMOL Molecular Graphics System (Schrödinger, LLC) using the PDB: 1GIA.

Plasmids and in vitro mRNA synthesis

Cloning of rat Go33 into pcDNA3 and pET28b has been described previously (22, 24). pcDNA3-Go33-HA [internally tagged; (40)] was from P. Wedegaertner (Thomas Jefferson University). Rat Go33 was cloned into the Eco RI/Sal I sites of the pGBK7 vector to generate the Gal4AD-Go33 fusion protein used in yeast two-hybrid experiments. A fragment of
Ric-8A corresponding to amino acids 12 to 491 was amplified from a plasmid (25) provided by S. Sprang (University of Montana) and cloned into the Nde I/Eco RI sites of the pGADT7 vector to generate the Gal4BD–Ric-8A fusion protein used in yeast two-hybrid experiments. A rat Gq13 construct internally tagged with yellow fluorescent protein (YFP) was generated by introducing the fluorescent protein in the b/c loop of the all-helical domain, which is a strategy previously described for Gq13 that preserves the native properties of the G protein (47). Briefly, YFP was inserted between S113 and A114 (b/c loop) of Gq13 by introducing silent mutations in pCDNA3.1(-)–Gq13 to create an artificial Afe I site in this location, followed by digestion and ligation of the sequence encoding for the fluorescent protein. pCDNA3.1-A1R, pCDNA3.1-VN-Gpγ2, pCDNA3.1-VC-Gβ1, pCDNA3.1-Gγ2, pCDNA3.1-Gβ1, and myc-ETaR-RLuc8 were provided by N. Lambert (Georgia Regents University) (20). pCDNA3.1-masGRK3ct-Nluc (21) and pcMin ETaR (42) were gifts from K. Martemyanov (Scripps Research Institute) and P. Polgar (Boston University), respectively.

Rat Gq13 was cloned into the Eco RI and Xho I sites of pCS2(+)(-) to generate the DNA template for in vitro mRNA transcription. Briefly, pCS2(+)(-)–Gq13 was linearized by digestion with Not I, and DNA purified by alkaline phenol/chloroform extraction followed by ethanol precipitation. Purified DNA (1 μg) was used as template for each mRNA in vitro transcription reaction with the SP6 mMessage mMachine Kit (Ambion). In vitro mRNA transcription reactions were treated by deixyribonuclease I to eliminate the template and mRNA purified by alkaline phenol/chloroform extraction, followed sequential precipitations in isopropanol and ethanol. Purified mRNAs were quantified spectrophotometrically and their quality checked in a 1% agarose/formaldehyde gel. mRNAs were diluted to the desired final concentrations and stored at −80°C.

Gq13 mutants were generated using specific primers (sequences available upon request) following the manufacturer's instructions (QuickChange II, Agilent). All constructs were checked by DNA sequencing.

X. laevis embryo manipulations

Frog studies were carried out with wild-type animals (Nasco) according to the Boston University Institutional Animal Care and Use Committee–approved protocol, in compliance with the Guide for the Care and Use of Laboratory Animals. Egg laying was induced by dorsal lymph injection of human chorionic gonadotropin (500 U) (Intervet). In vitro fertilization and embryo culture were carried out in 0.1× Marc's modified Ringer's medium as described (43). Staging was according to Nieuwkoop and Faber. In vitro transcribed mRNAs (30 or 60 pg) were injected equatorially in both dorsal blastomeres of four- or eight-cell stage embryos, which were subsequently incubated at 16°C. Embryos were fixed at late neurulation (stages 18 to 19) in MEMFA [100 mM Mops (pH 7.4), 2 mM EGTA, 1 mM MgSO4, 3.7% (v/v) formaldehyde], photographed, and subjected to phenotypic analysis. Embryos were observed using a Leica MZ6 dissection microscope and scored as “gravitational defect” when the neural tube was facing downward (18). Sagittal bisections of MEMFA fixed embryos at stage 15 were performed with a razor blade and photographed in 1× phosphate-buffered saline (PBS). All pictures were taken with a Canon XSi camera connected to the microscope.

For the analysis of Gq13 by immunoblotting, two embryos were resuspended in 60 μl of lysis buffer [20 mM Hepes (pH 7.2), 5 mM Mg(CH3COO)2, 125 mM K(CH3COO), 0.4% Triton X-100, and 1 mM dithiothreitol (DTT)], supplemented with a protease inhibitor cocktail (Sigma, catalog no. S8830) and homogenized by pipetting. After centrifugation for 10 min at 14,000g at 4°C, supernatants were supplemented with Laemmli sample buffer and boiled for 5 min.

Cell culture, transfections, and BRET assay

HEK293T cells were grown at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), 1% l-glutamine, and 5% CO2. All DNA plasmids were transfected using the calcium phosphate method in six-well plates, and BRET measurements were done 16 to 24 hours after transfection. Equal amounts (0.2 μg) of masGRK3ct-Nluc, VC-Gβ1, VN-Gpγ2, and GPCR (either A1R or ETaR) were transfected for BRET experiments. Gq13 and Gq4 were typically transfected at 1 μg of DNA per well. This amount of DNA was adjusted (1 to 4 μg) in some experiments to produce similar amounts of protein among different G proteins mutants and wild type. For the experiments investigating the dominant-negative effect of Gq13 on Gq4 activation, the plasmids were transfected at 1:8 ratio (0.12 μg:1 μg, Gq13;Gq4). BRET experiments were carried out and analyzed as described previously (20, 21) with minor modifications. Briefly, 16 to 24 hours after transfection, the cells were gently scrapped in PBS, centrifuged, and resuspended in Tyrode’s solution [140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 0.37 mM Na2HPO4, 24 mM NaHCO3, 10 mM Hepes (pH 7.4), and 0.1% glucose] at a density of 106 cells/ml. Cell suspensions (25 μl) were added to a white opaque 96-well plate (Opti-Plate, Perkin Elmer) and mixed with an equal volume of the NanoLuc substrate Nano-Glo (Promega, diluted 1:50). After 2 min of incubation, luminescence was measured at room temperature in a Synergy H1 plate reader (BioTek) at 460 ± 20 nm and 528 ± 10 nm. BRET signals were calculated as the ratio of the emission intensity at 528 ± 10 nm divided by the emission intensity at 460 ± 20 nm. For the kinetic experiments, BRET measurements were done every second. Basal BRET was measured for 30 s, after which adenosine or ET-1 was added to the well. With the exception of Fig. 2D, kinetic BRET data are presented as the increase in BRET (ΔBRET) for clarity. ΔBRET was calculated by subtracting the average of BRET signal before agonist stimulation (τ = 0 to 30 s) to every point of the time trace. An aliquot of the cell suspensions was processed for immunoblot analysis. Briefly, cells were pelleted, resuspended in lysis buffer [20 mM Hepes (pH 7.2), 5 mM Mg(CH3COO)2, 125 mM K(CH3COO), 0.4% Triton X-100, 1 mM DTT, supplemented with a protease inhibitor cocktail (Sigma, catalog no. S8830), and cleared by centrifugation at 14,000g for 4°C for 10 min. After centrifugation, supernatants were supplemented with Laemmli sample buffer and boiled for 5 min.

In vitro G protein biochemistry assays

Steady-state GTPase assays were performed using radiolabeled [γ-32P]GTP and measuring the release of [γ-32P]GDP at 30°C. GTPyS binding was determined by measuring intrinsic tryptophan fluorescence (excitation, 284 nm; emission, 340 nm) in a Hitachi F-4500 fluorescence spectrophotometer or by directly measuring the binding of radiolabeled [γ-33P]GTPyS at 30°C. Limited proteolysis assays were carried out by incubating purified G proteins or lysates of HEK293T cells expressing different G proteins with GDP, GTPyS, or GDP plus ALF4 at 30°C before adding trypsin. HPLC analyses of G protein nucleotide content were performed after exchanging the purified G proteins into nucleotide-free buffer and concentrating them to 50 μM. Nucleotide standards at 50 μM were prepared in the same buffer.

Immunoblotting

Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes, which were sequentially incubated with primary and secondary antibodies (goat anti-rabbit Alexa Fluor 680 and goat anti-mouse or IRDye 800, 1:10,000). The primary antibodies were used at the following dilutions: Gq13, 1:250; pan-Gβ1, 1:250; α-tubulin, 1:2500; HA, 1:1000; and rabbit myc, 1:1000. Infrared imaging of immunoblots was performed according
to the manufacturer’s protocols using an Odyssey Infrared Imaging System (LI-COR Biosciences). Images were processed using ImageJ software (National Institutes of Health (NIH)) and assembled for presentation using Photoshop and Illustrator software (Adobe).

**Protein purification**

His-tagged Gαi3 proteins were expressed in *E. coli* strain BL21(DE3) (Invitrogen) and purified as described previously (44). Briefly, bacterial cultures were induced with 1 mM isopropyl-β-D-thiogalactopyranoside overnight at 23°C. Pelleted bacteria from 1 liter of culture were resuspended in 25 ml of His lysis buffer [50 mM NaH2PO4 (pH 7.4), 300 mM NaCl, 10 mM imidazole, 1% (v/v) Triton X-100, 25 μM GDP, 1 μM leupeptin, 2.5 μM pepstatin, 0.2 μM aprotinin, and 1 mM phenylmethylsulfonyl fluoride]. After sonication (four cycles, with pulses lasting 30 s per cycle, and with 1-min interval between cycles to prevent heating), lysates were centrifuged at 12,000 g for 20 min at 4°C. Solubilized proteins were affinity-purified on HisPur Cobalt Resin (Pierce), eluted with imidazole, dialyzed against PBS, and buffer-exchanged/concentrated in 20 mM tris-HCl (pH 7.4), 20 mM NaCl, 1 mM MgCl2, 1 mM DTT, 10 μM GDP, and 5% (v/v) glycerol before storage at −80°C.

**Limited proteolysis assay with purified proteins**

This assay was carried out as described previously (44, 45) with minor modifications. Briefly, His-Gαi3 (0.4 mg/ml) was incubated for 90 min at 30°C in buffer [20 mM Na-Hepe (pH 8), 100 mM NaCl, 1 mM EDTA, 10 mM MgCl2, 1 mM DTT, and 0.05% (w/v) C12E10] supplemented with GDP (30 μM), GTPγS (30 μM), or GDP-αIF2α (30 μM GDP, 30 μM AlCl3, and 10 mM NaF). Then, trypsin was added to the tubes (final concentration, 80 μg/ml), and samples were incubated for 10 min at 30°C. Samples were rapidly transferred to ice, reactions were stopped by the addition of Laemmli sample buffer, and samples were incubated at 65°C for 5 min. Proteins were separated by SDS-PAGE and stained with Coomassie blue.

**Limited proteolysis assay with HEK293T cell lysates**

This assay was carried out as described previously (45) with minor modifications. HEK293T cells were transfected with plasmids encoding for wild-type Gαi3 or ACS mutants as described in the “Cell culture, transfections, and BRET assay” section. The amount of each plasmid required to achieve equal protein expression for wild-type and mutant Gαi3 was determined empirically in preliminary experiments. A quarter of the HEK293T cells from a well of a six-well plate was lysed in 48 μl of buffer [20 mM HEPES (pH 7.2), 5 mM MgCl2(CH3COO)2, 125 mM KCl(CH3COO), 0.4% Triton X-100, and 1 mM DTT] supplemented with 125 μM GDP or 125 μM GTPγS. Samples were vortexed, incubated in ice for 10 min, and centrifuged at 14,000g for 10 min at 4°C. The supernatants (40 μl) were incubated for 2 hours and 30 min at 30°C to allow the loading of nucleotide on the G protein. Trypsin (12.5 μg/ml) was added to the tubes and incubated at 30°C for 20 min. Reactions were stopped by the addition of 12 μl of Laemmli sample buffer and boiled for 5 min. The %= protection by GTPγS was calculated by quantifying the bands corresponding to “+trypsin/+GTPγS” condition divided by the band of “−trypsin” condition and multiplying the result by 100. To determine the specific protection of each ectopically expressed Gαi3 protein, the quantification values of the corresponding bands (that is, GTPγS-protected or total) of endogenous Gαi3 (that is, transfected with an empty vector) were subtracted before the calculation described above.

**Steady-state GTPase assay**

This assay was performed as described previously (44, 45). Briefly, reactions were initiated at 30°C by mixing assay buffer [20 mM Na-Hepe (pH 8), 100 mM NaCl, 1 mM EDTA, 2 mM MgCl2, 1 mM DTT, and 0.05% (w/v) C12E10] containing [γ-32P]GTP (1 μM, ~100 cpm/μmol) with an equal volume of His-Gαi3 (100 nM) in the same buffer. Duplicate aliquots (50 μl) were removed at 0, 2, 4, 6, 10, and 15 min, and reactions stopped with 950 μl of ice-cold 5% (w/v) activated charcoal in 20 mM H3PO4 (pH 3). Samples were centrifuged for 10 min at 10,000g, and 500 μl of the resultant supernatant were scintillation-counted to quantify the amount of [32P]Pi released. Data are presented as raw radioactivity counts.

**Measurement of GTPγS binding by intrinsic tryptophan fluorescence**

This assay was performed as described previously (45). Purified His-Gαi3 (1 μM) was equilibrated at 30°C in a cuvette with 1 ml of buffer [20 mM Na-Hepe (pH 8), 100 mM NaCl, 1 mM EDTA, 2 mM MgCl2, 1 mM DTT, and 0.05% (w/v) C12E10]. GTPγS (1.25 μM) was added to the cuvette after ~3 min, and the G protein activation rate was monitored by measuring the change in intrinsic fluorescence (excitation at 284 nm and emission at 340 nm) due to the structural rearrangement of the switch II tryptophan residue W211. Data were collected using a Hitachi F-4500 fluorescence spectrophotometer, background-corrected (buffer fluorescence), and presented as a ratio of the basal fluorescence (F/Fo).

**Measurement of radiolabeled GTPγS binding**

This assay was performed as described previously (44–46). Briefly, reactions were initiated at 30°C by mixing assay buffer [20 mM Na-Hepe (pH 8), 100 mM NaCl, 1 mM EDTA, 2 mM MgCl2, 1 mM DTT, and 0.05% (w/v) C12E10] containing [32P]GTPγS (1 μM, ~50 cpm/μmol) with an equal volume of His-Gαi3 (100 nM) in the same buffer. Duplicate aliquots (25 μl) were removed at the indicated time points, and binding of radioactive nucleotide was stopped by the addition of 2 ml of ice-cold wash buffer [20 mM tris-HCl (pH 8.0), 100 mM NaCl, and 25 mM MgCl2]. The quenched reactions were rapidly passed through BA85 nitrocellulose filters (Whatman). Filters were dried and subjected to scintillation counting. Data are presented as raw radioactivity counts.

**Nucleotide HPLC analysis**

HPLC analyses of G protein nucleotide content were performed as previously described (47). His-Gαi3 wild type and His-Gαi3 S47R mutant were exchanged into nucleotide-free buffer [20 mM tris-HCl (pH 7.4), 200 mM NaCl, 1 mM MgCl2, 1 mM DTT, and 5% (v/v) glycerol] and concentrated to 50 μM. Nucleotide standards at 50 μM were prepared in the same buffer. Acetonitrile was added to each protein sample or standard tube to obtain a final concentration of 7.5% (v/v); the mixture was boiled for 5 min and then centrifuged for 10 min at 20,000g to precipitate the denatured proteins. The supernatant was collected and used for the HPLC analysis. A Zorbax C-18 reversed-phase column (4.6 × 150 mm) filled with 3.5-μm silica (Agilent) was equilibrated with 100 mM KH2PO4 (pH 6.5), 10 mM tetra-butylammonium bromide (phase-transfer catalyst), 0.2% (w/v) NaN3, and 7.5% (v/v) acetonitrile using an Agilent 1260 Infinity Quaternary Pump flowing at 1 ml/min. Freshly prepared sample (15 μl) was loaded into 20 μl of HPLC loading loop. The samples were then injected onto the C-18 column and isocratically eluted at 1 ml/min. Absorbance of 280-nm wavelength light was performed by an Agilent 1260 infinity ultraviolet detector. Control standards of GDP and GTP were eluted at distinct retention times of 2.25 and 2.56 min, respectively.

**Coimmunoprecipitation**

This assay was performed as previously described (46). HEK293T cells were transfected in 10-cm dishes with plasmids encoding for untagged Gβ1 (1.2 μg), untagged Gγ2 (1.2 μg), myc-ETAR (6 μg), and wild-type
Go<sub>i3</sub>-YFP (internal b/c loop) or ACS mutants as described in the “Cell culture, transfections, and BRET assay” section. The amount of plasmid required to achieve equal protein expression for wild-type and mutant Go<sub>i3</sub> was determined empirically in preliminary experiments. HEK293T cells from a 10-cm plate were lysed in 1 ml of ice-cold buffer [20 mM Hepes (pH 7.2), 5 mM Mg(CH<sub>3</sub>COO)<sub>2</sub>, 125 mM K(CH<sub>3</sub>COO), 0.4% Triton X-100, and 1 mM DTT], vortexed, passed through a 30-gauge insulin syringe five times, and incubated in ice for 10 min. After centrifugation at 14,000 g for 10 min at 4°C, the supernatant was split into two tubes for each condition. Anti-myc (9E10) or control mouse IgG (2.5 µg; Santa Cruz Biotechnology) was added to each tube and incubated for 4 hours at 4°C with constant rotation. Protein G agarose beads (Thermo Scientific) were blocked with 5% bovine serum albumin (BSA) for 2 hours at room temperature, washed, added to each of the tubes containing lysates and antibodies, and incubated for 90 min at 4°C with rotation. Beads were then washed three times with wash buffer [4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 0.1% (v/v) Tween 20, 10 mM MgCl<sub>2</sub>, 5 mM EDTA, and 1 mM DTT], and proteins were eluted by incubation in Laemmli sample buffer for 10 min at 37°C.

### Immunofluorescence microscopy

This assay was performed as previously described (48). HEK293T cells were transfected with plasmids encoding for untagged G<sub>i3</sub>, untagged G<sub>y2</sub>, and Go<sub>i3</sub>-YFP (b/c loop internal) wild type or ACS mutants as described in the “Cell culture, transfections, and BRET assay” section except that they were seeded on poly-L-lysine–coated glass coverslips. The amount of plasmid required to achieve equal protein expression for wild-type and mutant Go<sub>i3</sub> was determined empirically in preliminary experiments. One day after transfection, cells were fixed with 3% paraformaldehyde for 30 min, permeabilized, blocked in PBS containing 10% normal goat serum and 0.1% Triton X-100 for 30 min, and then sequentially incubated with primary and secondary antibodies for 1 hour at room temperature. Antibody dilutions were as follows: primary mouse anti-GFP (B-2, Santa Cruz Biotechnology), 1:200; secondary goat anti-mouse Alexa 488 (Life Technologies), 1:300. Images were acquired with a Zeiss Axiovert 200 LSM510 laser scanning confocal microscope using a 63× oil immersion objective. All individual images were assembled for presentation using Photoshop and Illustrator software (both Adobe).

### Yeast two-hybrid assay

This assay was performed using Matchmaker Gold (Promega) according to the manufacturer’s instructions. Briefly, pGADT7-Ric-8A was transformed into the Saccharomyces cerevisiae haploid strain Y187, and pGBK7-Go<sub>i3</sub> (wild type or mutants) into the haploid strain AH109, using the lithium acetate method (49). Transformants were selected in synthetic defined (SD) medium plates lacking leucine (SD-Leu) and tryptophan (SD-Trp), respectively, and mated by co-inoculation of single colonies in YPD (yeast extract, peptone, and dextrose) medium and overnight incubation at 30°C. Mated diploid strains were selected by inoculation of 20 µl of the overnight cultures on SD-Leu-Trp medium. Individual colonies were inoculated into 3 ml of SD-Leu-Trp and incubated overnight at 30°C. This starting culture was used to inoculate 20 ml of SD-Leu-Trp at an optical density of 600 nm (OD<sub>600</sub> of 0.3. Exponentially growing cells (OD<sub>600</sub>~0.7 to 0.8; 4 to 5 hours) were pelleted to prepare samples for subsequent assays (see next sections).

### β-Galactosidase activity assay

This assay was performed as described previously (49) with minor modifications. Pellets corresponding to an OD<sub>600</sub> of 0.5 were washed once with PBS + 0.1% (w/v) BSA and resuspended in 200 µl of assay buffer [60 mM Na<sub>2</sub>PO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM MgCl<sub>2</sub>, 0.25% (v/v) β-mercaptoethanol, 0.01% (w/v) SDS, and 10% (v/v) chloroform] and vortexed. One hundred microliters was transferred to 96-well plates, and reactions were started by the addition of 50 µl of the fluorogenic substrate fluorescein di-β-D-galactopyranoside (final concentration, 100 µM). Fluorescence (excitation, 485 ± 10 nm; emission, 528 ± 10 nm) was measured every 2 min for 90 min at 30°C in a Synergy H1 plate reader (Biotek). Enzymatic activity was calculated from the slope of fluorescence (arbitrary units) versus time (minutes). At least three independent clones determined in duplicate were measured for each condition, and results were normalized (%) to the activity in cells expressing Ric-8A and wild-type Go<sub>i3</sub>.

### Preparation of yeast samples for immunoblotting

This procedure was performed as described previously (49, 50) with minor modifications. Briefly, pellets corresponding to an OD<sub>600</sub> of 5 were washed once with PBS + 0.1% BSA and resuspended in 150 µl of lysis buffer [10 mM tris-HCl (pH 8.0), 10% (w/v) trichloroacetic acid, 25 mM NH<sub>4</sub>OAc, and 1 mM EDTA]. Glass beads (100 µl) were added to each tube, and the samples were vortexed in a cold room for 5 min. Lysates were transferred by poking a hole in the bottom of the tubes followed by centrifugation onto a new set of tubes. The process was repeated after the addition of 50 µl of lysis buffer to wash the glass beads. Proteins were precipitated by centrifugation (20,000 g for 10 min) and resuspended in 60 µl of solubilization buffer [0.1 M tris-HCl (pH 11.0) and 3% SDS]. Samples were boiled for 5 min and centrifuged (20,000 g for 1 min), and 50 µl of the supernatant was transferred to new tubes containing 12.5 µl of Laemmli sample buffer and boiled for 5 min.

### Statistical analyses

Each experiment was performed at least three times. Data are presented as means ± SEM or as representative results of biological replicates. The error bars of the phenotype distributions shown in Fig. 2C were calculated as follows: SE = √(P(100 - P))/n, where P is the proportion (%) of the phenotype and n is the total number of embryos analyzed. Statistical significance between various conditions was assessed with Student’s t-test or Fisher’s exact test (for the Xenopus embryo assays). P < 0.05 was considered significant.

### SUPPLEMENTARY MATERIALS

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Fig. S1. ACS mutations in Go<sub>qα</sub> affect residues conserved across all G<sub>q</sub> proteins in humans.

Fig. S2. ACS-associated Go<sub>qα</sub> mutants bind to G<sub>i3γ</sub>.

Fig. S3. The subcellular localization of ACS-associated Go<sub>qα</sub> mutants is similar to that of wild-type Go<sub>qα</sub>.

Fig. S4. Go<sub>i3α</sub> and Go<sub>i3γ</sub> associate similarly with G<sub>i3γ</sub> subunits.

### REFERENCES AND NOTES


Acknowledgments: We are indebted to N. Lambert (Georgia Regents University) for providing critical reagents and extensive discussions for the BRET assay. We thank S. Sprang (University of Montana), K. Martemyanov (Scripps Research Institute), and P. Polgar (Boston University) for providing plasmids; E. Simmons (Boston University) for giving access to the spectrofluorimeter; and V. Trinkaus-Randall (Boston University) for providing access to the confocal microscope. We also thank A. J. McDonald and D. A. Harris (Boston University) for providing access to equipment and technical help for the HPLC experiments, and K. Steiling (Boston University) for assessment of the statistical analyses. Funding: This work was supported by NIH grants R01GM108733 and R01GM112631 and American Cancer Society grant RSG-13-362-01-TBE to M.G.-M.; NIH grant R01GM098367 and American Heart Association grant 10GRNT3010038 to I.D.; and a postdoctoral fellowship from the Hartwell Foundation to V.D.G. Author contributions: A.M., A.L., and M.G.-M. designed the study; A.M., A.L., K.P.-S., I.D., and M.G.-M. performed the experiments; I.D. contributed new reagents and analytic tools; A.M., A.L., K.P.-S., I.D., and M.G.-M. analyzed the data; and A.M. and M.G.-M. wrote the paper with input from all the authors. Competing interests: The authors declare that they have no competing interests.

Submitted 13 August 2015
Accepted 24 March 2016
Final Publication 12 April 2016
10.1126/scisignal.aad2429

Dominant-negative Gα subunits are a mechanism of dysregulated heterotrimeric G protein signaling in human disease


Sci. Signal. 9 (423), ra37.
DOI: 10.1126/scisignal.aad2429

Gq gets in the way of GqGq
Signaling by G protein–coupled receptors (GPCRs) regulates various aspects of development and adult physiology, and mutations in GPCR signaling pathway components cause disease. Some patients with auriculo-condylar syndrome (ACS), who have defects in craniofacial development, have mutations in the heterotrimeric G protein subunit GqGq. Developmental analysis of transfected Xenopus embryos and biochemical analysis in mammalian cells revealed that the GqGq mutations associated with ACS enable GqGq to bind inappropriately to the endothelin receptor ETAR and block the binding of another G protein, Gαq/11. Although able to bind ETAR, GqGq mutants lacked enzymatic activity, thereby preventing intracellular propagation of the endothelin signal. The findings show that dominant-negative mutations in one G protein can impair another and cause disease.